

## Controlled production of *Artemia* cysts in batch cultures

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### Abstract

The possibility to develop a routine batch culturing method for the production of cysts has been examined at laboratory scale.

The influence of abiotic and biotic parameters on the mode of reproduction has been studied and the following facts have been noted :

- cyclic low oxygen tensions induce haemoglobin synthesis with subsequent shift of the mode of reproduction from ovoviviparity to oviparity ;
- addition of ferric-EDTA stimulates haemoglobin synthesis ;
- a correlation exists between the color of the animals and the type of reproduction ;
- neither the food source nor the water salinity *per se* control the mode of reproduction. However, cysts produced at low salinity appear to have a low hatchability.

The effect on the hatchability of various processing techniques applied to the cysts produced has been tested. From these experiments it appears that :

- the cysts must be activated (dehydrated) to allow hatching at a subsequent hydration ;
- the method of dehydration influences to a large extent the viability of the cysts. Of the various methods tested out so far, drying at 40 °C or immersion in a saturated brine solution gave the best results.

A modified version of the classic air-water-lift operated raceway culturing-system is proposed as a standard method for the controlled production of cysts.

### Introduction

Although annually thousands of kilograms of *Artemia* cysts are harvested from nature, our knowledge of the phenomena which control cyst production is still very limited.

A factor common to all brine shrimp strains, parthenogenetic as well as bisexual, is that female *Artemia* are capable of producing either live offspring or inactive cysts. A detailed description of the reproductive mechanism and the different processes from oogenesis until deposition of the offspring can be found in Metalli and Ballardini (1972).

The eggs (oocytes) are formed in two string-like ovaria located on both sides of the digestive tract. When ripe, the eggs are transferred through the oviducts into the unpaired broodsac

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(uterus). At this stage bisexual females have to be fertilized to produce viable offspring. As soon as the embryonic process in the uterus has started, the production of new oocytes starts again. When the environmental conditions are "favorable" the mode of reproduction is ovoviviparous; the fertilized egg hereby undergoes the classical embryonic development and through blastulation and gastrulation the differentiation ends with the release of a free-swimming nauplius. Under influence of specific abiotic and biotic conditions ovoviviparity can, however, shift to oviparity with production of cysts; the embryonic development is hereby reversibly interrupted at the gastrula stage. Trehalose is stored as carbohydrate reserve (Clegg, 1962) and each gastrula is surrounded by a shell, impregnated with a haematine-like substance (Dutrieu, 1959) secreted by the active brown shell glands (Dutrieu, 1960; De Maeyer-Criel, 1978). These encysted gastrulae or so called cysts are finally released in the water.

In the literature much confusion exists about the mechanisms responsible for the shift from ovoviviparity to oviparity. Salinity was mentioned as the responsible factor by Abonyi (1915) and by Barigozzi (1939). Dutrieu (1960) correlated cyst production with haemoglobin synthesis. According to Ballardin and Metalli (1963) the mode of reproduction is controlled by environmental factors. D'Agostino and Provasoli (1968) reported that oviparity was induced by the food quality and/or quantity. Others refer to the presence of particular components in the culture medium or the food, e.g. iron (Baker, 1966) or chlorophyll (Dutrieu, 1960).

Previous research carried out in our laboratory (Sorgeloos, 1975) revealed a definite influence of low dissolved oxygen levels on the induction of oviparity. Unfortunately the technique applied was too complex to allow long term studies with many replicates excluding as such the possibility of statistical analyses.

For this reason we decided to use open aquaria in which the animals are maintained at densities of 1 000/l. Instead of maintaining low levels of dissolved oxygen, we have submitted the animals to cyclic drops of the oxygen concentration by applying a discontinuous aeration. As the exposures to critically low levels of oxygen were never maintained for long periods of time, the survival rates of the adult brine shrimp were kept high.

The present study deals with the influence of dissolved oxygen, iron, salinity, and type of food on the mode of reproduction of brine shrimp. Our specific goal was to develop a routine method which induces *Artemia* populations to shift from ovoviviparity to formation of cysts. Such a method can indeed become a very practical tool for the further study of the influence of abiotic and biotic parameters on characteristics of cysts. At the same time it may allow production of enough study material for comparative analyses of different *Artemia* strains.

## Materials and methods

All experiments were performed with the bisexual San Francisco Bay (California, USA) strain cultured in natural seawater, the salinity of which was increased with natural seasalts.

Three different culture tanks were used:

- 7 l rectangular aquaria equipped with one air-water-lift (AWL) placed in a corner;
- 40 l rectangular containers converted into AWL-raceways by placing a central partitioning and 4 AWL's (see Bossuyt and Sorgeloos, 1980 for more details on AWL-raceways);
- one 250 l rectangular AWL-raceway as described by Bossuyt and Sorgeloos (1980).

Reproduction tests were carried out with five different food sources. Stock suspensions were made up as follows :

- *Spirulina* : 50 g spray-dried *Spirulina* (commercially available from Sosa Texcoco, S.A., Mexico) homogenized in 250 ml seawater with a kitchen blender and diluted to 1 l with saturated brine ;
- *Spirulina*/yeast : 25 g *Spirulina* + 25 g bakers' yeast prepared in the same way as the *Spirulina*-suspension ;
- *Scenedesmus* : 15 g drum-dried *Scenedesmus* (made available by the "Kohlenstoff-biologische Forschungsstation", Dortmund, Fed. Rep. Germany) homogenized in 250 ml seawater with a kitchen blender, then grinded in a ball-mill (glass spheres 5 mm in diameter) for 12 hr and diluted to 1 l with saturated brine ;
- *Dunaliella* sp. cultured in natural seawater following De Pauw *et al.* (1978) ;
- rice bran : 100 g Ultrafine® micronized rice bran, defatted product (from the N.V. Brucoma, Ghent, Belgium) homogenized in 250 ml seawater with a kitchen blender and diluted to 1 l with saturated brine.

The following parameters were studied for qualitative and quantitative evaluation of the mode of reproduction of the brine shrimp :

- sex-ratio of 100 animals sampled at random from each culture tank ;
- fertility ratio : approximately 50 females were examined under the dissection microscope for full ovaria, oviducts and/or uterus ; the ratio of the number of mature females to the total number of females observed was used to quantify the fertility of the population. Experiments started if this figure was above 50 % and lasted until it dropped below 50 % ;
- reproductive capacity : 10 females with a full uterus were transferred to individual vials containing 20 ml culture medium. Once a day the vials were checked for deposited cysts or nauplii and the number of offspring was recorded. All females released their offspring within 5 days and the ratio of oviparous to ovoviviparous females as well as the average number of cysts and nauplii produced per female was calculated ;
- color-code of Chow (1968), as a rough estimate of the haemoglobin concentration in *Artemia* :

0 : no color  
+ : light pink  
+ + : light pink, few spots red  
+ + + : red  
+ + + + : dark red.

## Experimental results and discussion

### CONTINUOUS VERSUS DISCONTINUOUS AERATION

A 5-weeks reproduction test was performed with 3 weeks-old adult *Artemia* in two 7 l aquaria at 90 ‰ salinity and 28 °C. The animals were fed with *Spirulina* (20 ml stock suspension per day per aquarium). Every week the medium was renewed.

One aquarium was aerated continuously (average oxygen concentration : 6.0 mg/l). In the other one a 10 min interruption was applied automatically once every hour ; this resulted in a

cyclic drop of the oxygen level to 4.5 mg/l. which is close to the critical level for induction of oviparity (Sorgeloos, 1975).

The results are summarized in Table IA. After two to three reproductive cycles, the animals kept under cyclic oxygen fluctuations shifted from ovoviviparity to oviparity. Of a total of 67 broods, 71% consisted of cysts. The observation that under discontinuous aeration the *Artemia* turn red (as compared to pale in the control) is probably related to the increased haemoglobin production. Gilchrist (1954) and Dutrieu (1960) observed indeed that at low oxygen levels brine shrimp are stimulated to produce haemoglobin. Haematin, a decomposition product of the latter respiratory pigment is then secreted by the shell glands at the moment of cyst production (Fautrez and Fautrez-Firlefijn, 1971).

TABLE I  
Influence of different factors on the mode of reproduction of *Artemia*.  
A : continuous versus discontinuous aeration ;  
B : Fe-EDTA addition in media aerated continuously ;  
C : Fe-EDTA addition in media aerated discontinuously

		Number of broods	Mortality rate (%)	% oviparous broods	Color of the animals
A	Continuous aeration	70	20	39	Pale
	Discontinuous aeration	67	45	71	Light pink
B	Fe-EDTA	100	20	67	Dark pink
	Control	100	30	33	Pale
C	Fe-EDTA	100	15	96	Red
	Control	100	30	74	Pink

#### THE EFFECT OF Fe-EDTA ON CYST PRODUCTION

Research carried out in California (USA) revealed a positive correlation between the presence of iron in the medium, increased haemoglobin synthesis, and cyst production (Baker, 1966 ; Chow, 1968). The experimental technique used was, however, difficult to keep under control and often resulted in high mortalities.

To check the influence of iron addition to the medium we carried out experiments in two 7 l aquaria tests (similar culturing conditions as described for the series with continuous aeration). One aquarium was progressively (over a period of 1 week) enriched with Fe-EDTA<sup>2</sup> to a final concentration of 20 mg Fe-EDTA/l medium.

Our results, summarized in Table IB, confirm the theory of Baker (1966) : over a 7 weeks test-period the dominant mode of reproduction was oviparous : 67% versus 33% in the

<sup>2</sup> Fe EDTA stock solution (Baker, 1966)

Fe Cl <sub>3</sub> 6H <sub>2</sub> O	2.4 g
Na <sub>2</sub> EDTA	1.86 g
aq. dest.	1 000 ml.

control series. The presence of chelated iron appears to facilitate haemoglobin synthesis: the animals indeed had a higher haemoglobin content in the iron enriched medium than in the control series. As has been reported earlier by Gilchrist (1954), Dutrieu (1960) and Anonymous (1978), the survival rate of dark-colored animals is higher than that of pale *Artemia*. In this regard it is interesting to note that we often observed "surface respiration" (Baker, 1966; Moore and Burn, 1968; Horne, 1971) in pale animals but never in dark pink or red adults.

#### THE COMBINED EFFECT OF DISCONTINUOUS AERATION AND Fe-EDTA ADDITION ON CYST PRODUCTION

Two 7 l aquaria, both with discontinuous aeration but one enriched with Fe-EDTA were set up in conditions identical to those previously described. After an adaptation period of 7 days the percentage of oviparous females was determined twice a week over a 5 weeks period. From the data obtained (Table IC) it is clear that cyclic oxygen stresses and the presence of chelated iron, when acting in concert, stimulate cyst production by 20 to 30% respectively compared to their single effect.

#### STATISTICAL ANALYSIS OF THE EFFECT OF CYCLIC OXYGEN STRESSES AND Fe-EDTA ADDITIONS ON CYST PRODUCTION

Prior to run this experiment we have determined the minimal oxygen and maximal Fe-EDTA level that should be applied to maximize cyst production.

Three oxygen levels have been evaluated in 7 l aquaria, set up in identical conditions as described earlier, all enriched with 20 mg Fe-EDTA:

- aquarium 1: continuous aeration (average oxygen concentration of 6.2 mg/l)
- aquarium 2: every hour, a 10 min interruption of the aeration (minimal oxygen concentration: 4.0 mg/l)
- aquarium 3: every hour, a 30 min interruption of the aeration (minimal oxygen concentration: 2.5 mg/l)

From the results of a 5 weeks reproduction test (Table II) it appears that a cyclic drop of the dissolved oxygen level to 4 mg/l is sufficient to induce cyst production. A further decrease of the oxygen concentration has no beneficial effect: on the contrary the animals often concentrated at the water surface even during periods of oxygenation. Survival rate dropped to 40% and the fertility and size of the broods was smallest in the aquarium with the highest oxygen stress.

TABLE II  
Influence of three types of aeration on the mode of reproduction

Aeration mode	% oviparous females
Continuous	33
Discontinuous (minimum 4 mg/l)	78
Discontinuous (minimum 2.5 mg/l)	89

In the next experiment we have examined the effect of 5 concentrations of Fe-EDTA : 10, 15, 20, 25, and 30 mg/l respectively. Five 7 l aquaria were set up in standard culture conditions with continuous aeration. The results in Table III indicate that addition of either 25 or 30 mg Fe-EDTA/l give a maximal cyst production, with no specific differences between the two series.

TABLE III  
Influence of five concentrations of Fe-EDTA on the mode of reproduction

Fe-EDTA concentration (mg/l)	% oviparous females
10	22
15	32
20	32
25	68
30	71

Four 40 l raceways were used to test the combined effects of cyclic oxygen stresses (down to 4 mg/l) and Fe-EDTA additions (25 mg/l) :

- A : continuous aeration, no iron addition
- B : continuous aeration, iron addition
- C : discontinuous aeration, no iron addition
- D : discontinuous aeration, iron addition.

Each raceway contained about 40 000 *Artemia* in 90 ‰ seawater at 28 °C. Once adult the daily feeding consisted of 100 ml *Spirulina* suspension per raceway. Ten consecutive broods were studied during an experimental period of 2 months. Data on the following parameters are summarized in Table IV : % survival, dissolved oxygen concentration, sex-ratio, fertility ratio, cysts to nauplii or C/N-ratio, and color-code of Chow (1968). The data for the C/N-ratios were submitted to a variance analysis with two factors and replicates within the sub-classes. The average were compared using Duncan's Multiple Range Test (Snedecor and Cochran, 1967).

The results of this experiment confirm our previous findings : the highest percentage of oviparous females is found in the presence of extra-iron. The average C/N-ratio for the tests with addition of iron and discontinuous aeration was significantly different from all other combinations at the 0.01 level (Table V, A to D). A definite correlation was furthermore noted between the color of the animals and their mode of reproduction. Our observations confirm the earlier findings of Clegg (personal communication) that females with white shell glands produce nauplii, those with brown shell glands desposit cysts.

#### DIAPAUSE DESACTIVATION IN *ARTEMIA* CYSTS

Cysts deposited in raceway cultures were harvested and cleaned from large debris by washing through a 350 µm screen and recuperated on a 200 µm filter screen.

TABLE IV

Reproductive characteristics of *Artemia* in 10 consecutive broods

as influenced by Fe-EDTA addition and mode of aeration

A : continuous aeration, iron addition ; B : continuous aeration, no iron addition ;

C : discontinuous aeration, iron addition ; D : discontinuous aeration, no iron addition

	% survival	Dissolved oxygen (mg/l)	Sex-ratio	Number of females from a random sample of 100 adults with full ovaries/full oviducts/full uterus	% fertility	C/N ratio	Color code
A	98	6.3	1.43	10/ 4/ 4	40	0.08	+
	91	6.3	1.38	14/10/ 8	55	0.1	+
	97	6.3	1.60	10/12/ 1	54	0.17	+ / + +
	96	6.3	1.52	12/10/ 2	55	0.24	+ / + +
	96	6.3	1.60	14/16/22	70	0.32	+ / + +
	95	6.2	1.56	16/ 9/10	60	0.60	+ +
	94	6.2	1.50	17/10/ 4	52	0.70	+ +
	93	6.2	1.54	12/ 9/12	55	0.72	+ +
	93	6.2	1.58	16/ 8/ 2	59	1.00	+ +
B	99	6.3	1.27	10/ 4/72	29	0.06	0
	98	6.3	1.33	3/ 1/ 3	13	0.07	0
	96	6.3	1.50	10/ 3/11	40	0.04	0
	95	6.3	1.52	12/11/ 4	45	0.08	0
	95	6.3	1.38	10/ 4/11	43	0.06	0
	94	6.2	1.40	12/ 6/ 4	38	0.05	0
	94	6.2	1.47	12/ 9/10	53	0.08	0
	93	6.0	1.50	14/ 6/11	52	0.10	0
	92	6.0	1.46	11/10/ 4	44	0.06	0
C	99	5.6	1.2	12/ 4/ 6	49	1.06	+ / + +
	98	5.4	1.42	14/ 6/ 4	40	1.17	+ +
	96	4.9	1.63	14/10/ 8	52	2.30	+ +
	95	4.6	1.63	12/ 6/ 9	44	3.40	+ +
	95	4.4	1.58	10/ 4/ 3	44	4.00	+ + / + + +
	93	4.2	1.60	14/ 6/ 9	47	17.0	+ + +
	93	3.8	1.62	16/ 9/ 6	50	14.0	+ + +
	93	3.7	1.62	19/16/ 6	66	24.0	+ + + / + + + +
	91	3.4	1.61	16/ 6/10	51	29.0	+ + + +
D	96	5.4	1.3	16/ 4/ 8	49	0.09	0
	94	5.4	1.63	12/10/ 4	42	0.70	0
	93	4.8	1.60	3/ 1/ 3	11	0.06	0 / +
	90	4.6	1.50	10/ 4/ 2	30	0.03	0 / +
	88	4.3	1.54	6/10/ 4	36	0.07	+
	82	4.0	1.59	14/ 6/ 4	39	0.90	+
	76	3.8	1.62	16/ 4/ 8	45	1.20	+
	74	3.7	1.60	14/ 8/ 4	42	1.00	+
	70	3.4	1.52	18/ 4/ 8	48	1.00	+

TABLE V

Analysis of variance and Duncan's Multiple Range Test for significant differences in C/N-ratios of *Artemia* submitted to combined effects of Fe-EDTA and mode of aeration (see Table IV)

	d.f.	Average SS	F <sub>w</sub>
SS total	35	—	—
SS Fe-EDTA	1	243.26	8.85**
SS aeration	1	261.79	9.53**
SS interaction	1	209.86	7.64**
SS replicates	8	32.23	—
SS error	24	27.48	—
Standard error averages : 1.7474			
Duncan's Multiple Range Test			
C-B*			
C-A* D-B*			
C-D* D-A A-B			

When incubated in optimum hatching conditions (Sorgeloos, 1980) less than 3% of these cysts gave birth to nauplii. This confirms the theories of Morris (1971) and Clegg (1974) that the hatching metabolism in *Artemia* cysts can only be initiated when the cyst's diapause has been desactivated. Following Dutrieu (1960) and Morris and Afzelius (1967) this dormancy state can be overcome by dehydration of the cysts.

We have tested this hypotheses in the following experiment : after different time intervals of exposure to saturated brine<sup>3</sup> (4, 6, 12, 24, and 48 hr respectively) the percentage hatching efficiency was determined by incubation of 100 cysts (three parallels) in natural seawater in stoppered plastic vials kept in continuous movement on a rotating axle (Sorgeloos *et al.*, 1978). From the hatching results recorded after 48 hr incubation at 25 °C (Fig. 1), it appears that the hatching efficiency increases as a function of the dehydration time in brine. Although the hatching percentage does not significantly increase after prolongation of the exposure to brine for 2 days, the maximal hatching obtained is still lower than the optimal hatching efficiency data reported in the literature for San Francisco Bay cysts. We do not know if this is related to the incomplete water removal in brine – according to Clegg (personal communication) cysts exposed to saturated NaCl solutions still contain  $\pm 20\%$  water – or to specific effects of the rearing conditions (biotic and/or abiotic parameters) on particular characteristics of the deposited cysts.

Dehydration of *Artemia* cysts can be achieved by various methods. We have analysed the hatching efficiency of cysts harvested from the same raceway cultures but subjected to different drying conditions for varying exposure times :

- oven-drying at 30, 40, 50, and 60 °C respectively ;
- dehydration in excicator over dry CaCl<sub>2</sub> ;
- dehydration in saturated brine solution.

<sup>3</sup> Since upon direct transfer to saturated brine a high percentage of laboratory produced cysts implode, the cysts were initially dehydrated for 1 hr in 115 ‰ brine and then transferred to saturated brine of 280 ‰.



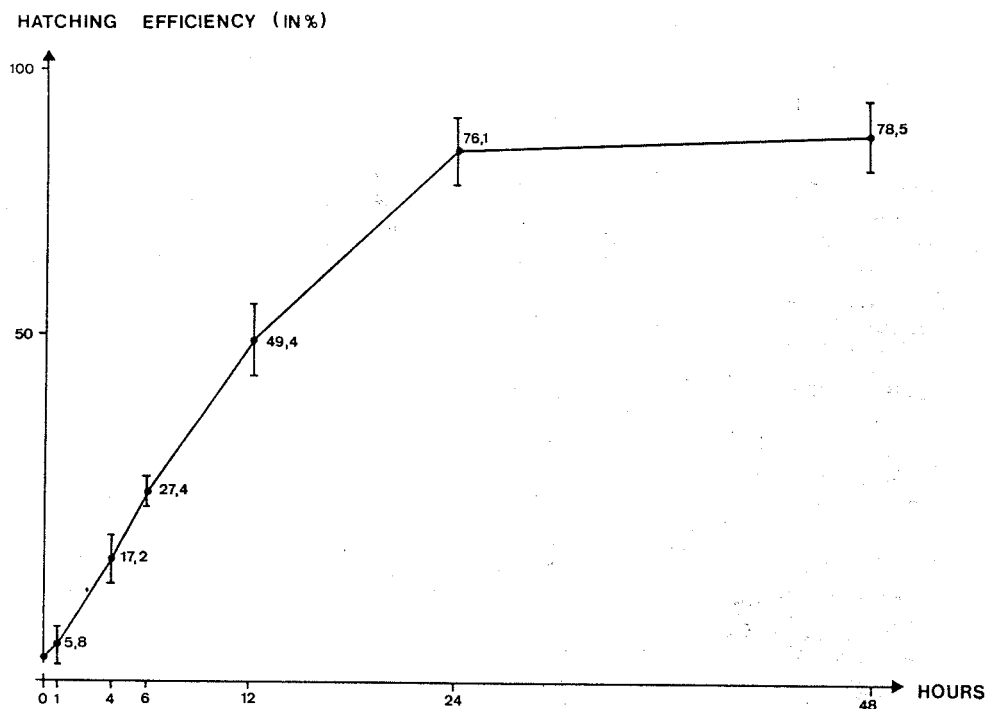


FIG. 1. Influence of dehydration time in saturated brine on the hatching efficiency of laboratory produced cysts.

The results are represented graphically in Fig. 2. Dehydration for 24 hr at 40 °C in an oven or at room temperature over  $\text{CaCl}_2$  or in a saturated brine solution assures a  $\pm 80\%$  hatchability of the treated cysts. As could be expected exposure to temperatures above 40 °C negatively influence the viability of cysts (Sorgeloos *et al.*, 1976). We are wondering if the low hatching scores recorded for 48 hr drying over  $\text{CaCl}_2$  might not be related to the extremely low water content of these cysts; this is at least the explanation which Iwasaki (1958) gave for her finding that cysts dried over  $\text{CaCl}_2$  are more sensitive than untreated cysts. The best hatching result of 81% obtained with oven-dried cysts is significantly better than the 76% recorded for the brine-activated cysts. Further water removal from 20% to less than 10% thus assures a better desactivation of the diapause mechanism.

#### THE EFFECT OF VARIOUS FOOD SOURCES ON CYST PRODUCTION

Five 7 l aquaria were set up with 7 000 adult *Artemia* each in 90 ‰ seawater at 28 °C. No cyst-inducing conditions were created. The following diets known to be good *Artemia* foods (Sorgeloos, 1975; Sorgeloos *et al.*, 1980) were administered at specific daily rations:

- 50 ml *Dunaliella* suspension at  $1.2 \cdot 10^6$  celles/ml
- 10 ml *Spirulina* suspension

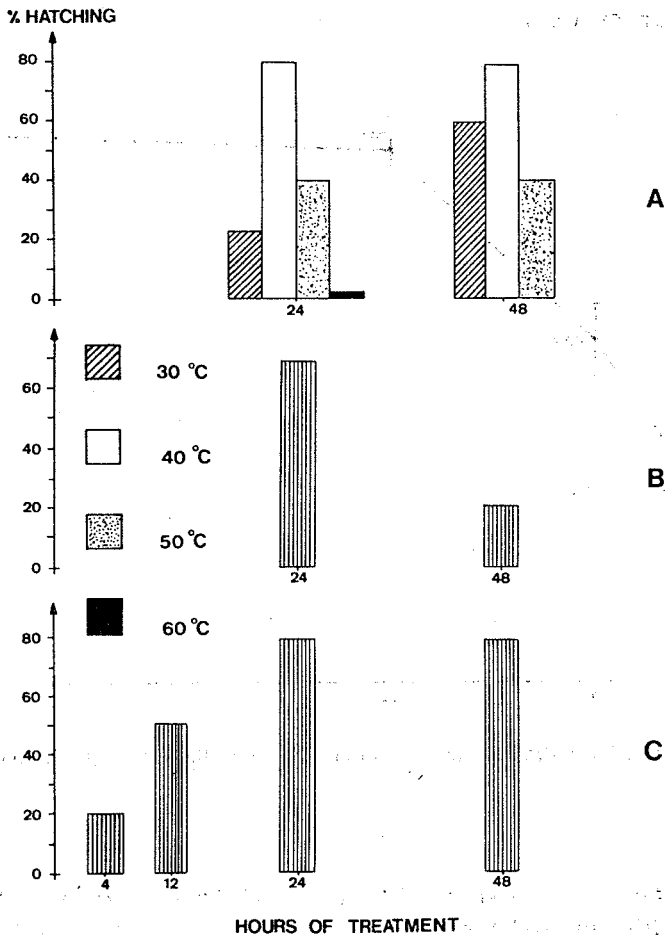


FIG. 2. Influence of different drying methods on the hatching percentage of cysts harvested from raceway cultures. A : oven drying ; B : drying in excicator over  $\text{CaCl}_2$  ; C : dehydration in saturated brine.

- 10 ml *Scenedesmus* suspension
- 10 ml *Spirulina*-bakers' yeast suspension
- 10 ml rice bran suspension.

The percentage oviparous females totalized for each diet over a 1 month reproductive period for the various diets is summarized in Table VI. It appears from these data that no typical cyst-inducing food could be found among the diets tested. In the given set of experimental conditions *Dunaliella* and *Scenedesmus* proved to be less efficient in stimulating the oviparous mode of reproduction than *Spirulina* and rice bran. The results obtained with rice bran is in contradiction with Dutrieu (1962)'s theory that chlorophyll in the diets is a *conditio sine qua non* for oviparity.

TABLE VI

Influence of four algal diets and one food without chlorophyll on the mode of reproduction

Diet	% oviparous females
<i>Dunaliella</i>	15
<i>Scenedesmus</i>	12
<i>Spirulina</i>	28
<i>Spirulina</i> /yeast	25
Rice bran	30

We have repeated these reproduction experiments with rice bran-fed, respectively *Spirulina*-fed *Artemia* in cyst-inducing conditions (discontinuous aeration and Fe-EDTA additions). The data obtained (Fig. 3) confirmed our previous results : over 90 % of the females shifted to oviparity and no significant difference could be noted between the two diets.

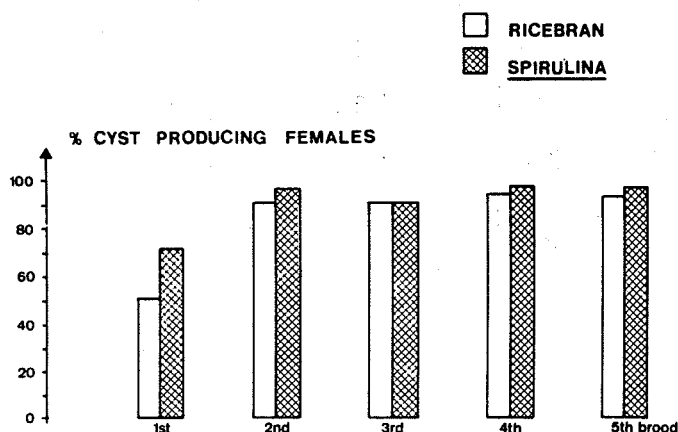


FIG. 3. Influence of two different diets on the mode of reproduction of brine shrimp subjected to cyst-inducing conditions.

#### THE INFLUENCE OF TWO DIETS, TWO TEMPERATURES AND THREE SALINITIES ON THE MODE OF REPRODUCTION IN *ARTEMIA*

In order to define optimal temperature/salinity combinations for routine production of *Artemia* cysts in small raceways, the following experiment was performed : twelve 40 l raceways containing 40 000 adult *Artemia* each, were maintained under cyst-inducing conditions (discontinuous aeration, Fe-EDTA-addition). The percentage oviparous females was followed over 10 reproductive cycles in function of the following variables :

- temperature : 20 and 28 °C
- salinity : 30, 90, and 180 ‰
- diet : *Spirulina* : 40 ml stocksuspension per day  
rice bran : 50 ml.

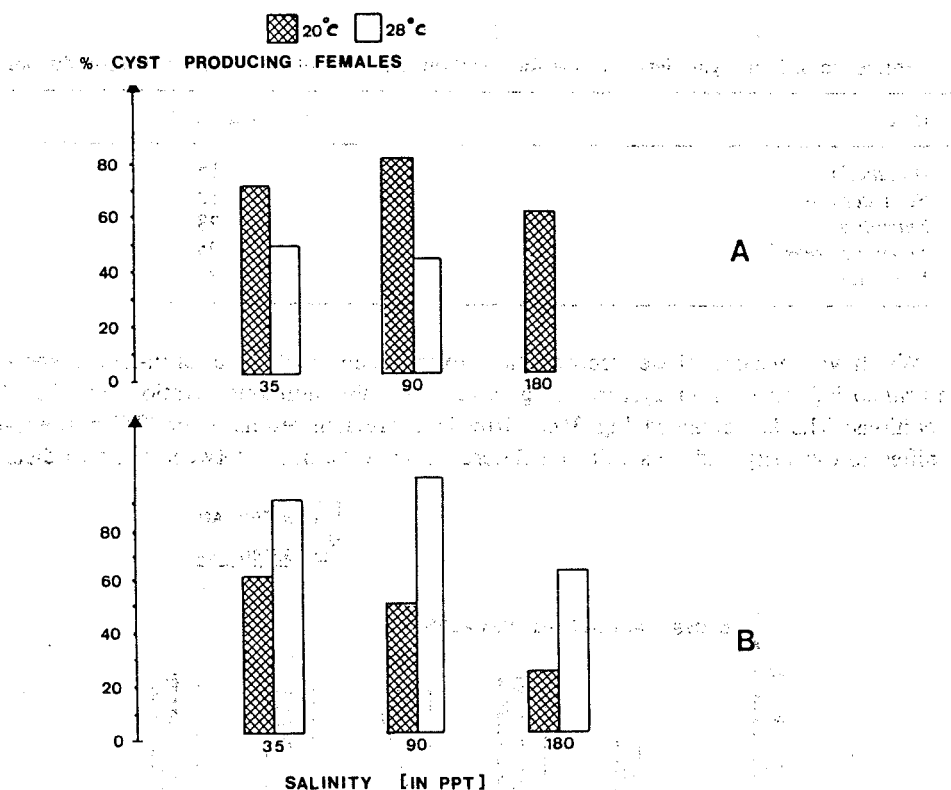


FIG. 4. Influence of two diets (A : *Spirulina* ; B : ricebran), two temperatures and three salinities on the mode of reproduction in *Artemia*.

Cysts harvested from the various experimental combinations were activated in brine solution and incubated at 28 °C in 35 ‰ seawater during 48 hr to determine their hatching efficiency. The results (Fig. 4) indicate that with *Spirulina* maximal cyst production occurred at 20 °C, whereas with rice bran it was at 28 °C. The 180 ‰ cultures were difficult to maintain due to clogging of the aeration lines by cristallization of salt; This phenomenon led to the loss overnight of combination 28 °C/180 ‰/*Spirulina*. Although no statistical difference existed between the 35 ‰ and 90 ‰ parallels, the hatching quality of the cysts produced at 35 ‰ (Fig. 5) turned out to be poor and this for both the 20 °C and 28 °C series. A similar problem in hatching cysts produced in natural seawater was noted by Tobias (personal communication) in his flow-through cultures with live algae. Further research is needed here to find out which factor(s) cause(s) the low viability in these cysts and to define the optimal conditions for activation or hatching in these cysts.

The largest number of cyst-producing females was recorded for the rice bran combination at 28 °C and 90 ‰ ; in this particular set of conditions almost 30 % more cysts were deposited than in the corresponding *Spirulina* series at 20 °C.

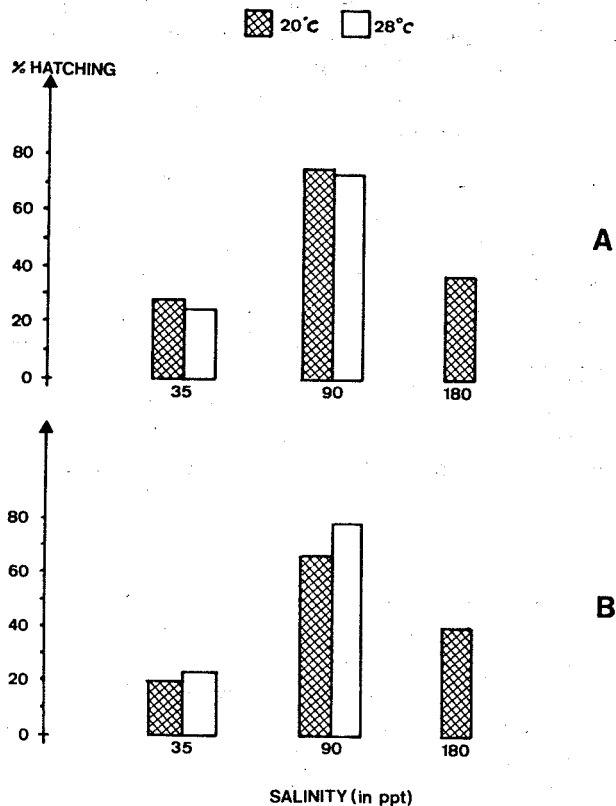


FIG. 5. Hatchability of *Artemia* cysts produced in different combinations of temperature, salinity and diet (A : *Spirulina* ; B : rice bran) after 48 hr incubation in seawater of 35 ‰ salinity at 28 °C.

#### Standard procedure for controlled cyst production in batch systems

From the information gained in this study the following standard procedure for routine production of *Artemia* cysts in batch systems can be advised :

- brine shrimp are raised to adulthood in densities of 1 ind./l at 28 °C in 90 ‰ seawater using a rice bran suspension as sole source of food. Good growth and survival results are obtained by using "air-water-lift (AWL)-raceways" as described by Bossuyt and Sorgeloos (1980) ;
- after 4 weeks culturing, Fe-EDTA is gradually added in order to reach a concentration of 25 mg/l after 1 week ;
- from the 5th week onwards, the animals are submitted to cyclic oxygen stresses which should not exceed a minimal level of 4 mg/l ;
- within another 1 to 2 weeks, over 90 % of the females will switch to oviparity ;
- the deposited cysts which mostly float in a medium of 90 ‰ salinity should be harvested as frequently as possible ; their cleaning and separation from faecal pellets, respectively empty

shells and other debris is carried out following the two steps processing procedure in brine and tap water as described by Sorgeloos *et al.* (1978). Attention must be paid, however, that prior to the brine treatment the cysts should be exposed to a salinity of 115 ‰ for at least 1 hr; the cysts should be dried in an oven at 35 to 40 °C for 24 hr and consequently stored under vacuum or in a nitrogen atmosphere.

We have applied this standard procedure in our laboratory in a 250 l raceway. An electromagnetic valve, activated by an electric clock and connected to the air-inlet was used to impose cyclic oxygen stresses. A plate-separator was coupled to the raceways to harvest and separate cysts from faeces. With this system we were able to harvest an average production of 1 g dry cysts/day over a production period of 1 month.

The same technique has been applied in the SEAFDEC Aquaculture Department in the Philippines (Sorgeloos, 1978), with manual interruption of the aeration a few times per day to induce dissolved oxygen drops. Food consisted of a mixture of *Spirulina* and ricebran. Cysts were harvested both in the culture tank and from a plate-separator. Over a 2 week test-period the daily harvest from a 1 m<sup>3</sup> raceway with only 100 adults per liter averaged 5.5 g.

## Conclusion

Controlled production of *Artemia* cysts in small scale systems appears to be technically feasible. The key to success is the application of cyclic oxygen stresses in media enriched with Fe-EDTA. A positive correlation appears between the presence of Fe-EDTA in the medium, cyclic oxygen stresses, red coloration of the animals related to the degree of haemoglobin synthesis, and cyst production. These findings corroborate the theory of Dutrieu (1960) that *Artemia* females use their haemoglobin as a basic element for cyst shell formation. Low oxygen levels are known to induce synthesis of haemoglobin in order to facilitate respiration. It appears from our studies that the need for chlorophyll in the diet as an essential factor for the induction of oviparity (Dutrieu, 1962) can be eliminated by addition of chelated iron to the medium.

Since this study was performed with brine shrimp from the San Francisco Bay (California, USA) strain, the results should not be extrapolated to all *Artemia* strains. Considerable variations may indeed exist with regard to specific quantitative factors such as optimal temperature, dosage of Fe-EDTA, sensitivity for oxygen stresses, etc.

It also should be made clear that the procedure worked out cannot be considered, even by scaling up, as an alternative for cyst production in nature. The harvest for aquaculture purposes of tons of cysts produced naturally in salt ponds will always be much cheaper than any "artificial" cyst production. However, the technique of controlled cyst production opens many interesting perspectives in the study of various aspects of the reproductive biology in *Artemia*. It provides the biochemist with a standardized research material of known origin to unravel the mechanisms which induce the shift in the reproductive behavior and allows for the first time the study of the influence of different abiotic and biotic parameters on the characteristics of standard cysts. Application of this technique with various strains, either harvested from natural populations or produced from cross-breeding, will allow to compare their reproductive behavior and to study the heritability of particular characteristics. Finally, in those cases where only a very limited quantity of cysts or animals of a particular strain are

available, the technique described will be a valuable tool in the production of more research material either for direct testing or as inoculation material for further production in nature.

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